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Bacterial community succession during *in situ* uranium bioremediation: spatial similarities along controlled flow paths

Chiachi Hwang[1,7], Weimin Wu[2], Terry J Gentry[3], Jack Carley[4], Gail A Corbin[1],
Sue L Carroll[4], David B Watson[4], Phil M Jardine[4], Jizhong Zhou[5,8], Craig S Criddle[2]
and Matthew W Fields[6,7,8]

[1]*Department of Microbiology, Miami University, Oxford, OH, USA;* [2]*Department of Civil and Environmental Engineering, Stanford University, Stanford, CA, USA;* [3]*Department of Crop and Soil Sciences, Texas A & M University, College Station, TX, USA;* [4]*Environmental Sciences Division, Oak Ridge National Laboratory, Oak Ridge, TN, USA;* [5]*Institute for Environmental Genomics, University of Oklahoma, Norman, OK, USA;* [6]*Department of Microbiology, Montana State University, Bozeman, MT, USA;* [7]*Center for Biofilm Engineering, Montana State University, Bozeman, MT, USA and* [8]*Virtual Institute of Microbial Stress and Survival (http://vimss.lbl.gov/)*

Bacterial community succession was investigated in a field-scale subsurface reactor formed by a series of wells that received weekly ethanol additions to re-circulating groundwater. Ethanol additions stimulated denitrification, metal reduction, sulfate reduction and U(VI) reduction to sparingly soluble U(IV). Clone libraries of SSU rRNA gene sequences from groundwater samples enabled tracking of spatial and temporal changes over a 1.5-year period. Analyses showed that the communities changed in a manner consistent with geochemical variations that occurred along temporal and spatial scales. Canonical correspondence analysis revealed that the levels of nitrate, uranium, sulfide, sulfate and ethanol were strongly correlated with particular bacterial populations. As sulfate and U(VI) levels declined, sequences representative of sulfate reducers and metal reducers were detected at high levels. Ultimately, sequences associated with sulfate-reducing populations predominated, and sulfate levels declined as U(VI) remained at low levels. When engineering controls were compared with the population variation through canonical ordination, changes could be related to dissolved oxygen control and ethanol addition. The data also indicated that the indigenous populations responded differently to stimulation for bioreduction; however, the two biostimulated communities became more similar after different transitions in an idiosyncratic manner. The strong associations between particular environmental variables and certain populations provide insight into the establishment of practical and successful remediation strategies in radionuclide-contaminated environments with respect to engineering controls and microbial ecology.

Introduction

Uranium contamination from extraction processes for nuclear weapons production remains a significant environmental problem, and the use of depleted uranium and other heavy metals in non-nuclear weapons is an additional environmental hazard. Depleted uranium is weakly radioactive and can damage mammalian kidneys because of heavy metal toxicity (Craft *et al.*, 2004), and the oxidized

form of uranium (that is, U(VI)) is soluble, and thus mobile in groundwater. Particular microorganisms can use metals and metalloids such as U(VI) as an electron acceptor (Lloyd, 2003), and the formation of U(IV) through reduction forms a less soluble precipitate that is much less likely to contaminate water supplies. Populations within the community respond differently to disturbances and processes, and it is the cumulative effect of multiple populations directly or indirectly connected that facilitates overall activity. Therefore, work is needed to understand the relationships between biotic and abiotic parameters in the context of bioremediation.

The Field Research Center (FRC; http://www.esd.ornl.gov/orifrc/) on the Oak Ridge Reservation in eastern Tennessee was established by the US

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Department of Energy to evaluate *in situ* strategies for the long-term treatment of radionuclide wastes. Mixed wastes are difficult and expensive to remediate effectively with current physical and chemical technologies, but bioremediation with indigenous microorganisms holds promise as a cost-effective and comparatively unobtrusive technology for *in situ* remediation (Iwamoto and Nasu, 2001). Many aspects of bioremediation must be better understood for successful and efficient applications including the complex relationships between the microorganisms involved in contaminant removal, changes in pollutant concentration, and geochemical and hydrological conditions.

The study site had an initial pH of approximately 3.5 and was contaminated with nitrate, toxic metals and organic contaminants, which resulted from waste disposal over a 30-year period (Figure 1). During the course of the above study, we characterized changes in bacterial community structure over time and space: at the injection well (intermittent ethanol additions); at the extraction well used to capture and recycle water; at wells within the treatment zone (between the injection and extraction well) and at up- and down-gradient wells. The results demonstrate how system hydraulics and engineering control measures can impact structure and function and alter community dynamics within U(VI)-reducing communities.

Materials and methods

Site description and biostimulation tests

The well and pump system consisted of an outer groundwater recirculation loop with a nested inner recirculation loop (Figure 1). The outer loop, with recirculation between wells FW103 and FW024, created a hydraulic barrier that reduced the amount of contaminated groundwater from entering the nested inner loop (Figure 1). Within the inner loop, the carbon/electron donor (ethanol) was injected weekly into well FW104 and extracted at well FW026, and FW101 and FW102 (13.7 m depth) were the stimulated zones. Ethanol was selected based upon earlier batch microcosm experiments with field samples (Wu et al., 2006a). The recirculation flow rates in the inner loop and the outer loop (from FW103 to FW024) were 0.45 l min^{-1}. Additional clean water was injected at well FW024 at 0.7–0.9 l min^{-1} to minimize entry of ambient groundwater, increase the pH, and remove nitrate. Detailed descriptions are available elsewhere (Hwang et al., 2006; Luo et al., 2006; Wu et al., 2006a, 2007, http://www.esd.ornl.gov/orifrc/). Stimulation of bacterial growth with ethanol began on day 137 (7 January 2004) and ended on day 754 (15 September 2005) (Wu et al., 2006b, 2007). The ethanol solution (industrial grade, 88.1% ethanol, 4.7% methanol and 7.2% water w/w) had a chemical oxygen

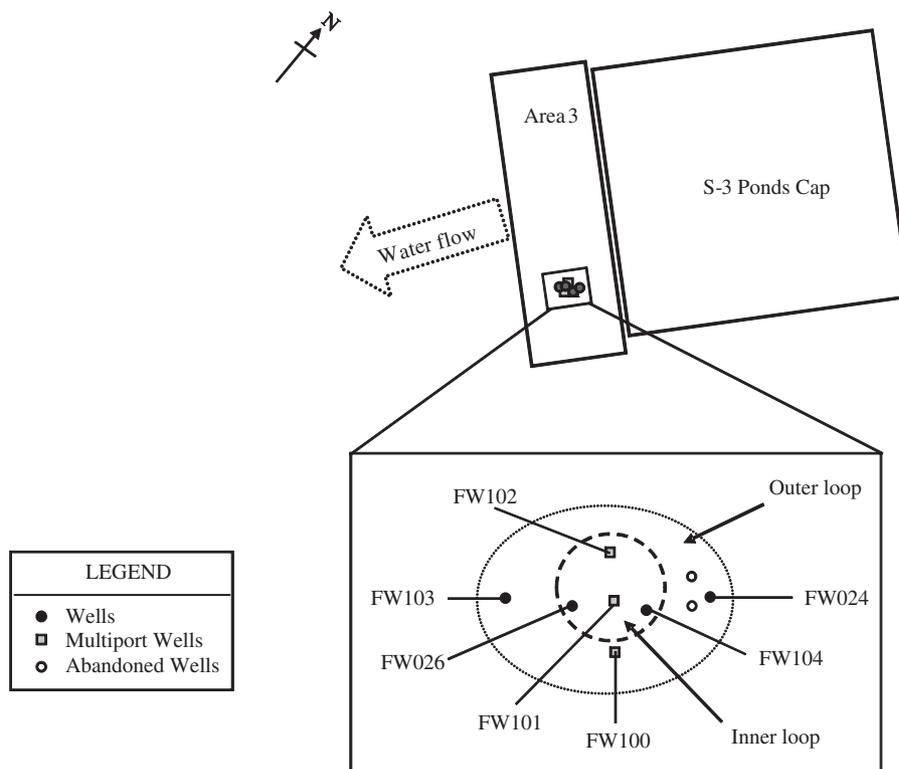**Figure 1** Schematic map of the former waste ponds at the Oak Ridge-Field Research Center in Oak Ridge, TN, USA and the inset shows location of the six sampled wells during the establishment of conditions conducive for uranium bioreduction. The substratums of the sampling sites were similar and consisted of shale with inner-embedded limestone that has been weathered to clay and silty saprolite. Water flow denotes groundwater flow.

demand (COD)-to-weight ratio of 2.1. The solution was prepared at a COD of 6.9–9.8 g l^{-1} in a storage tank and injected weekly at FW104 over a 48-h period to give 120–150 mg l^{-1} of COD at FW104 except during a starvation experiment from days 713 to 754. The groundwater temperature fluctuated between 12 and 21 °C because of seasonal variation over the course of the study (data not shown). For later time points, dissolved oxygen (DO) was maintained at low levels (<0.03 mg l^{-1}) as described earlier (Wu *et al.*, 2007).

Analytical methods
Groundwater samples were collected from the injection, extraction and monitoring wells to track biogeochemical changes. Anions (nitrate, sulfate, bromide and so on) were analyzed with an ion chromatograph (Dionex DX-120, Sunnyvale, CA, USA) as described earlier (Wu *et al.*, 2006b). COD was used to monitor the consumption of electron donor (ethanol) and metabolic by-products (for example, acetate). COD, sulfide and Fe^{2+} were determined using a Hach DR2000 spectrophotometer (Hach Company, Loveland, CO, USA). U(VI) concentration was determined by kinetic phosphorescence analysis using a KPA-11 analyzer (ChemCheck Instruments, Richland, WA, USA).

Sample collection, DNA extraction and DNA purification
Groundwater was collected (approximately 2–5 l), filtered and the filters stored at −80 °C until DNA could be extracted. Biomass collected on the filters (polycarbonate, 0.2 μm, Nucleopore, Whatman, Florham Park, NJ, USA) was washed, vortexed and centrifuged with 1× phosphate-buffered saline before DNA extraction. Samples were suspended in 1× phosphate-buffered saline and the cells were disrupted through two cycles of freeze–thaw and grinding in sterile sand as described earlier (Zhou *et al.*, 1996). DNA was extracted and purified from all samples with a PowerSoil DNA Isolation Sample Kit (MO BIO, Carlsbad, CA, USA). On the basis of total cell counts and an assumed DNA amount per cell (∼5 fg), recovery efficiency was estimated to be 30–40% for all samples.

SSU rRNA gene PCR amplification, cloning and sequence determination
SSU rRNA gene sequences were amplified by PCR with universal bacterial primers FD1 (5′-AGAGTTT GATCCTGGCTCAG-3′) and 1540R (5′-AAGGAGGT GATCCAGCC-3′) as described earlier (Fields *et al.*, 2005, 2006). Each 20-μl PCR contained approximately 5–10 ng μl^{-1} of DNA, 200 nM of each primer, 10 μl of Bulls Eye Taq DNA polymerase 2.0 mix (Midwest Scientific, St Louis, MO, USA) and an adjusted volume of sterilized water. The lowest number of PCR cycles was determined for each sample to minimize PCR-induced artifacts, and five PCRs were combined before cloning. PCR conditions were as follows: denaturation at 94 °C for 2 min, the number of optimal cycles of 94 °C for 30 s, annealing at 58 °C for 1 min, extension at 72 °C for 1 min and a final extension at 72 °C for 7 min. Negative PCR controls without DNA template were run concurrently for each sample. PCR products (5 μl) mixed with DNA loading buffer (2 μl) were visualized by agarose gel electrophoresis in a 0.8% agarose Tris-acetate EDTA gel stained with ethidium bromide. Clone libraries were constructed through a methodology to minimize possible biases introduced by PCR as described earlier (Fields *et al.*, 2005, 2006) and the predominant population in this study was verified with quantitative PCR (qPCR).

The PCR products were purified with a spin column purification kit (Promega, Madison, WI, USA). Clone libraries were constructed with the TOPO TA pCR2.1 cloning kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Transformants from each clone library were checked for inserts by PCR with M13 primers (30 cycles of the PCR conditions described above except an annealing temperature of 60 °C and a final extension time of 10 min). The amplification products were analyzed by agarose gel electrophoresis.

PCR products from each clone were purified with a Montage PCR$_{u96}$ plate according to the manufacturer's instructions (Millipore, Bedford, MA, USA). DNA sequences were determined with a Big-DYE Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions at 1:4 dilution with an internal sequencing primer (529r) as described earlier (Fields *et al.*, 2005, 2006). Sequence reaction products were run on an ABI 3730 DNA sequencer.

DNA sequence analysis
DNA sequences were analyzed with Sequencher (v.4.0; Gene Codes Corporation, Ann Arbor, MI, USA) and vector sequences were removed. The sequences were analyzed for chimeras with Chimera-Check (Cole *et al.*, 2003) and Bellerophon (Huber *et al.*, 2004), and the chimeric sequences were removed from further analyses. SSU rRNA gene sequence identification was performed by comparison with known sequences in GenBank with the BLASTN server (http://www.ncbi.nlm.nih. gov/blast/Blast.cgi) and the RDP (http://rdp.cme. msu.edu/index.jsp). Unique SSU rRNA gene sequences were parsed into operational taxonomic units (OTUs) based on 97% sequence identity. All clone sequences and reference sequences were aligned with Clustal W (Thompson *et al.*, 1994) and phylogenetic trees were constructed with the neighbor-joining method and Jukes–Cantor distance model with bootstrap values of 500 replicates within MEGA v.4.0 (Tamura *et al.*, 2007).

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Quantitative PCR
On the basis of clone library data, quantification of bacterial DNA for *Desulfovibrio* and *Geobacter* populations was performed with a Rotor-Gene 3000 Real-Time PCR Detection System (Corbett Life Sciences, Sydney, Australia) and Rotor-Gene Interface software (v6.0.31; Corbett Life Sciences). PCR primers Dsv691F/Dsv826R and Geo494F/Geo825R were used as described earlier (Holmes *et al.*, 2002; Fite *et al.*, 2004). Amplification reactions were carried out with Platinum Quantitative PCR Super-Mix-UDG following the manufacturer's instructions (Invitrogen). DNA concentrations were approximately 100 ng per reaction, and the DNA was purified before qPCR by the cetyl trimethyl ammonium bromide method. Primer concentration was optimized with genomic DNA from *Desulfovibrio vulgaris* and *Geobacter metallireducens*. The following temperature profiles were used for amplification: one cycle at 95 °C for 1 min, followed by 40 cycles of denaturation at 95 °C (10 s), primer annealing (15 s) at 62 °C and extension (30 s) at 72 °C. Quantification was performed with standard curves made from known concentrations of DNA. Melt curve analyses were performed by heating the PCR mixtures from 60 to 95 °C (1 °C per cycle of 10 s) with simultaneous measurements of SYBR Green signal intensities.

Statistical analysis
Rarefaction curves, OTU richness, bacterial community diversity measures (for example, Shannon–Weiner (H') index, 1/Simpson's ($1/D$) index and Chao1) and sample coverage for each clone library were determined by the software program DOTUR (Schloss and Handelsman, 2005) with a 3% difference in nucleic acid sequences, and the evenness index (E) was calculated within Krebs software as described earlier (Brown and Bowman, 2001). The H' index considers the equitability of the OTU distribution, $1/D$ also considers both richness and abundance and Chao1 is a non-parametric estimation of OTU richness. However, composition and abundance may not be completely epitomized by clone distribution; therefore, measurements and indices were used for relative comparisons (Yan *et al.*, 2003; Fields *et al.*, 2006). Comparisons between clone libraries were made with different approaches. LIBSHUFF (http://www.arches.uga.edu/~whitman/libshuff.html) analysis was used to construct a pairwise comparison of the SSU rRNA gene libraries as described earlier (Fields *et al.*, 2006). UPGMA and a neighbor-joining method were used to construct phylogenetic trees with distance matrices based upon correlation values of ΔC_{xy} values with MEGA v4.0 (Tamura *et al.*, 2007).

Ordination analyses
Overall variation in bacterial community composition throughout the treatment process was charac-terized by Jackknife environmental cluster analysis and principal coordinates analysis within the Uni-Frac software package (Lozupone *et al.*, 2007) according to specified instructions given at the UniFrac website (http://bmf2.colorado.edu/unifrac/index.psp), and correspondence analysis (CA), detrended correspondence analysis and canonical correspondence analysis (CCA) were performed with Canoco v4.5 (Microcomputer Power Inc., Ithaca, NY, USA). Detrended correspondence analysis, an indirect gradient analysis technique that detrends by segments, was used to obtain the length of the gradient in the species data to allow for the selection of the appropriate method (unimodal or linear). When comparisons were made among all wells, species data were reported in percentage OTUs. When bacterial community profiles were compared between the injection and the two inner bioreduction wells only, species data were reported as percentage in bacterial families instead of percentage of OTUs. Detrended correspondence analysis revealed that the two data sets exhibited a unimodal (gradient length = 6.74 and 3.51 for comparison between all wells and wells of the bioreduction zone, respectively) response to the explanatory variables. Thus, CCA, a direct gradient analysis, was used to examine the relationship between bacterial community profiles and environmental factors (that is, well characteristics, time, pH, sulfate, nitrate, and subsurface manipulations). CCA has been shown to provide a flexible and meaningful constrained ordination of ecological species abundance data with environmental variables (Anderson and Willis, 2003). Partial CCA (pCCA) was also used to discern patterns related to one set of variables while controlling for a different set of variables (covari-ables; see Table 3).

A total of 14 explanatory variables were used when communities from all wells were analyzed: six dummy variables were assigned to characterize spatial locations (extraction, injection, and the inner and outer wells); four dummy variables were assigned to characterize engineered conditions of the subsurface (residual denitrification, ethanol stimulation without DO control, ethanol stimulation with DO control and ethanol limitation with DO control); and pH, sulfate, nitrate and time were each considered as a variable. A total of 14 explanatory variables were used when communities from the injection and the two inner bioreduction wells were compared: three dummy variables were assigned to characterize spatial locations, four dummy variables for the engineered conditions of the subsurface as described above and pH, COD, uranium, nitrate, sulfide, sulfate and time were each considered as a variable.

Variance partitioning as described by Anderson and Gribble (1998) and Muylaert *et al.* (2000) was performed to determine which variables (that is, environmental, spatial or temporal) could best explain the observed distribution of species compo-

sition (Table 3). The P-values for the canonical axes were produced by Monte Carlo permutation tests to determine the significance of microbial community changes in relation to environmental variables. CCA does not depend on parametric distributional assumptions and performs well with skewed species distributions (Palmer, 1993). Thus, species data were not transformed and explanatory variables were standardized.

Results

Biostimulation and U(VI) reduction
Nitrate concentrations dropped to below 0.05 mM after day 540 once stimulation was initiated on day 137 (Figure 2). U(VI) concentrations decreased to below US EPA Maximum Contaminant Limit for drinking water (0.03 mg l^{-1}) after day 615 in FW102. During ethanol injection, the COD concentration in wells FW104, FW101 and FW102 increased initially and then decreased as ethanol was consumed (Table 1). However, COD levels decreased to near zero when ethanol was not added from days 713 to 754. The same trend was observed for sulfide concentrations and the opposite was observed for sulfate levels. Uranium concentrations in the injection well, FW104, decreased less rapidly than the two inner bioreduction wells, FW101 and FW102.

A slight rebound in uranium concentration occurred after ethanol injection stopped, but U(VI) levels remained below 0.2 mg l^{-1}.

Tracer tests with bromide indicated that the outer and inner loops were hydraulically connected. Approximately 9% of the water injected at well FW104 flowed to the outer-loop extraction well FW103, and approximately 17% of the water injected to the outer-loop well FW024 flowed to the inner-loop extraction well FW026 (Luo et al., 2007). As a result, small amounts of ethanol injected into the inner-loop leaked to the outer-loop wells.

Bacterial diversity (richness and relative abundance)
A total of 23 clone libraries were constructed for the six tested wells. Approximately 80–100 clones (90 ± 7; Table 2) were analyzed for each library and approximately 2000 SSU rRNA gene sequences (approximately 500 bp in length) were analyzed to determine population distributions over the tested time period. When a 3% OTU cutoff was used, rarefaction curves for most clone libraries approached saturation (Supplementary Figure S1), and the percent coverage for sampled diversity ranged from 63% to 93% (data not shown). The relative bacterial diversity and OTUs declined over time in well FW024 (Table 2). Although the number of sampled OTUs was initially stable in the injection
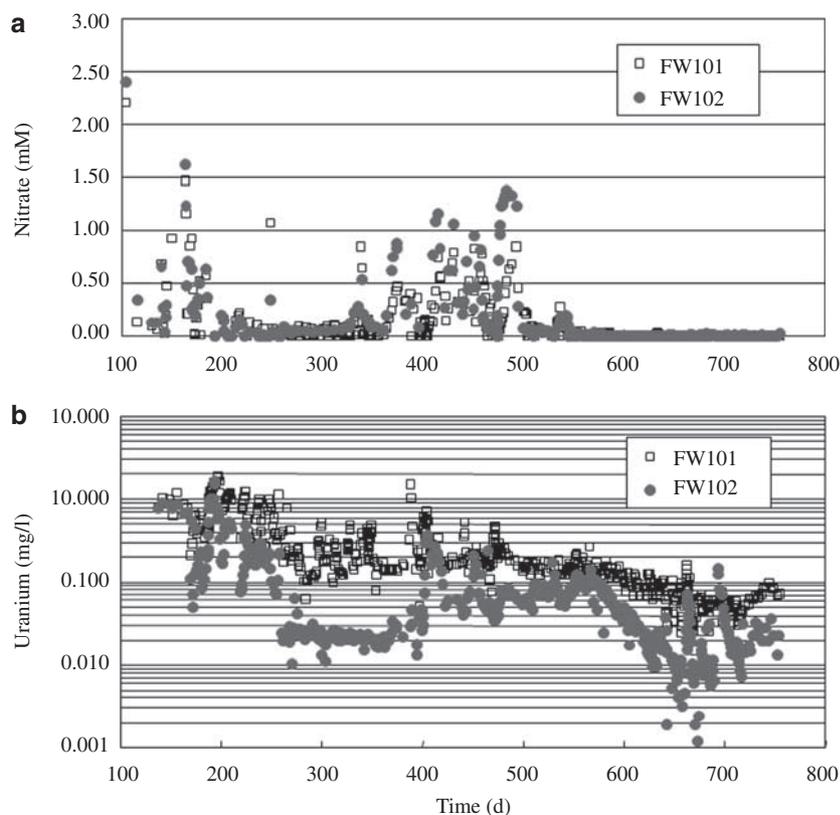

Figure 2 Nitrate (**a**) and uranium U(VI) concentrations (**b**) in groundwater of monitoring wells (FW101 and FW102) over time in the bioreduction zone.

Table 1 Chemical properties of groundwater during the bioremediation process

Sample number	Well	Days	COD (mg l⁻¹)	Sulfate (mg l⁻¹)	Sulfide (mg l⁻¹)	pH	Nitrate (mM)	U(VI) (mg l⁻¹)
1	FW024	166	9	31.6	ND	5.89	0.198	0.50
2		535	7	26.3	ND	5.92	0.190	0.07
3		712	<2	130	ND	5.75	0.015	0.017
4	FW104	166	40.0	88.0	ND	6.16	0.811	1.053
5		535	137	42.0	0.00	6.88	0.038	0.174
6		641	137	40.6	1.52	5.93	0.0017	0.165
7		712	119	58.7	9.34	5.70	<0.001	0.142
8		746	4.00	111	0.01	5.77	0.011	0.137
9	FW101-2	166	17.0	89.3	ND	6.51	0.211	0.791
10		278	3.00	47.6	NA	6.43	0.0047	0.152
11		535	70.0	39.3	2.23	6.35	<0.001	0.186
12		558	95.0	17.9	2.74	5.88	<0.001	0.175
13		622	96.0	2.23	7.48	6.03	<0.001	0.068
14		641	108	2.78	9.90	6.02	0.001	0.042
15		726	8.00	101	0.84	6.00	<0.001	0.062
16	FW102-2	166	9.00	76.5	ND	6.33	0.711	0.696
17		278	2.00	27.3	NA	5.97	0.047	0.027
18		622	51.0	8.66	3.37	6.24	<0.001	0.021
19		670	59.0	9.72	7.35	6.25	<0.001	0.002
20		726	12.0	96.3	0.66	6.24	<0.001	0.023
21	FW026	535	32.0	41.6	0.04	6.11	0.085	0.242
22	FW103	535	10	27.1	ND	6.12	0.147	0.083
23			<2	135	ND	5.97	0.021	0.05	<2

Abbreviations: COD, chemical oxygen demand; ND, not determined; NA, not applicable.
NA denotes data not analyzed and ND denotes not determined (below detection limit).

Table 2 Characteristics and diversity estimates for SSU rRNA gene clones from groundwater samples at different time points

Sample number	Well	Days	Number of clones[a]	OTU[b]	H'[c]	1/D[d]	Chao-1[e]	Evenness[f]
1	FW024	166	83	34	3.13	17.2	53.1	0.83
2		535	84	31	2.79	10.4	73.0	0.76
3		712	90	20	2.26	6.20	27.3	0.68
4	FW104	166	84	24	2.58	9.35	69.5	0.74
5		535	81	27	2.66	8.21	40.0	0.76
6		641	80	24	2.93	14.3	29.2	0.80
7		712	97	25	2.89	16.7	26.2	0.83
8		746	89	41	3.37	28.4	95.2	0.84
9	FW101–2	166	84	16	1.97	4.56	19.0	0.64
10		278	85	16	2.24	7.83	19.0	0.73
11		535	83	33	3.10	17.1	50.0	0.83
12		558	98	22	2.25	5.21	29.5	0.66
13		622	98	35	3.00	14.3	70.0	0.78
14		641	87	20	2.55	11.4	32.0	0.78
15		726	92	23	2.25	5.09	42.5	0.64
16	FW102–2	166	103	28	2.30	4.81	47.4	0.63
17		278	100	42	3.16	15.12	150.8	0.79
18		622	98	39	3.16	18.35	126.8	0.81
19		670	101	30	2.57	8.43	72.0	0.69
20		726	95	52	3.61	32.13	101.6	0.93
21	FW026	535	84	36	3.10	16.60	72.1	0.80
22	FW103	535	85	24	2.33	6.46	48.0	0.67
23		747	90	28	2.49	6.337	58.6	0.67

[a]Number of clones sequenced from each library.
[b]Operational taxonomic unit (OTU) based on partial SSU rRNA gene sequences (⩾97%).
[c]Shannon–Weiner index, higher number represents higher diversity.
[d]Reciprocal of Simpson's index, higher number represents higher diversity.
[e]Chao-1 estimates.
[f]Evenness index.

well FW104, an increase in sampled bacterial diversity and evenness (Table 2) was eventually observed. However, in the two bioreduction wells, FW101 and FW102, a fluctuation in diversity and evenness was observed from days 166 to 726. In general, diversity increased during periods of ethanol addition. Diversity decreased in FW101 before implementation of DO control (maintenance of low DO, <0.03 mg l⁻¹), but recovered to levels comparable to those of FW102 by day 622.

Table 3 Summary of calculation and results of CCA conducted to partition the percent variance in the distribution of bacterial populations explained by environmental (engineering controls and subsurface geochemistry), temporal and spatial variables

Steps	Description	All of the wells			Wells of the bioreduction zone		
		Sum of canonical eigenvalues	% Variance explained[a]	P-values[b]	Sum of canonical eigenvalues	% Variance explained[a]	P-values[b]
(1)	Environmental variables explanatory (CCA)	2.450	33.40	0.002	1.722	66.90	0.008
(2)	Spatial variables explanatory (CCA)	1.711	23.30	0.334	0.383	14.90	0.178
(3)	Temporal variables explanatory (CCA)	0.628	8.60	0.002	0.435	16.90	0.002
(4)	Environmental variables explanatory, space as covariable (pCCA)	2.459	33.50	0.010	1.613	62.70	0.018
(5)	Environmental variables explanatory, time as covariable (pCCA)	2.265	30.80	0.006	1.416	55.00	0.072
(6)	Non spatially or temporally structured environmental variation (pCCA)	2.347	32.00	0.002	1.290	50.10	0.114
(7)	Spatial variables explanatory, environment as covariable (pCCA)	1.720	23.40	0.118	0.275	10.70	0.284
(8)	Spatial variables explanatory, time as covariable (pCCA)	1.721	23.40	0.020	0.404	15.70	0.036
(9)	Non-environmentally or temporally structured spatial variation (pCCA)	1.803	24.60	0.030	0.278	10.80	0.264
(10)	Temporal variables explanatory, environment as covariable (pCCA)	0.443	6.03	0.028	0.130	5.05	0.382
(11)	Temporal variables explanatory, space as covariable (pCCA)	0.638	8.70	0.002	0.456	17.70	0.002
(12)	Non-environmentally or spatially structured temporal variation (pCCA)	0.527	7.20	0.038	0.133	5.20	0.332
(13)	Spatially structured environmental variation[c]	−0.009	−0.12	—	0.109	4.20	—
(14)	Temporally structured environmental variation[d]	0.185	2.52	—	0.306	11.90	—
(15)	Unexplained variation[e]	2.646	36.00	—	0.444	17.25	—

Abbreviation: pCCA, partial canonical correspondence analysis.
[a]The percentage of explained variation is calculated as a proportion of the total inertia of the species data (all wells: 7.343; wells of bioreduction zone: 2.574).
[b]P-values based on block Monte Carlo permutation test, as described in Materials and methods.
[c]Spatially structured environmental variation = ([1]−[4])/total inertia × 100.
[d]Temporally structured environmental variation = ([1]−[5])/total inertia × 100.
[e]Unexplained variation = total inertia−(sum of canonical eigenvalues in steps [1]+[7]+[12]) × 100.

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Bacterial composition and structure
For the purposes of this study, composition equates to richness and structure represents diversity. Phylogenetic analysis of the sampled SSU rRNA gene sequences revealed that bacterial communities in the groundwater changed in composition and structure throughout the different stages of treatment (Figures 3a–d). Initially, β-*Proteobacteria* predominated, but as ethanol stimulation continued, sequences indicative of other bacterial phyla such as δ-*Proteobacteria*, *Actinobacteria*, *Acidobacteria* and *Firmicutes* became prevalent (Figure 3a). At day 166, β-*Proteobacteria* was the major group in the FW024, FW104, FW101 and FW102 libraries. However, as diversity increased, the β-*Proteobacteria* populations fluctuated in FW104, FW101 and FW102 (Figure 3a). Predominant sequences within the β-*Proteobacteria* were closely related to *Herbaspirillum*, *Zooglea*, *Dechloromonas*, *Ferribacterium*, *Hydrogenophaga*, *Curvibacter* and *Acidovorax* spp. (Supplementary Figure S2B and C). Both *Zooglea*

and *Acidovorax* sequences and isolates have been observed earlier in FRC groundwater (Fields *et al.*, 2005).

FW103 at day 535 was predominated by *Rhodocyclaceae* populations (49%), and the sequences were similar to clones from FW024 on the same sampling day. Like FW024, the sampled community in FW103 also shifted to *Comamonadaceae* populations (51%) at day 747 (Figure 3b). Most of the clone populations in the *Comamonadaceae* family had 98% sequence identity to *Rhodoferax ferrireducens*, whereas others had 98% sequence identity to *Acidovorax delafieldii* (Supplementary Figure S2B and C). Clone libraries from FW104 and FW101 were predominated by an *Oxalobacteraceae* population at day 166 and the clone sequences had ⩾97% sequence identity to *Herbaspirillum* spp. (AJ012069) (Supplementary Figure S2B). FW104 shifted from predominance by an *Oxalobacteraceae* population (40%) in the residual denitrification phase to predominance by *Rhodocyclaceae* population (most
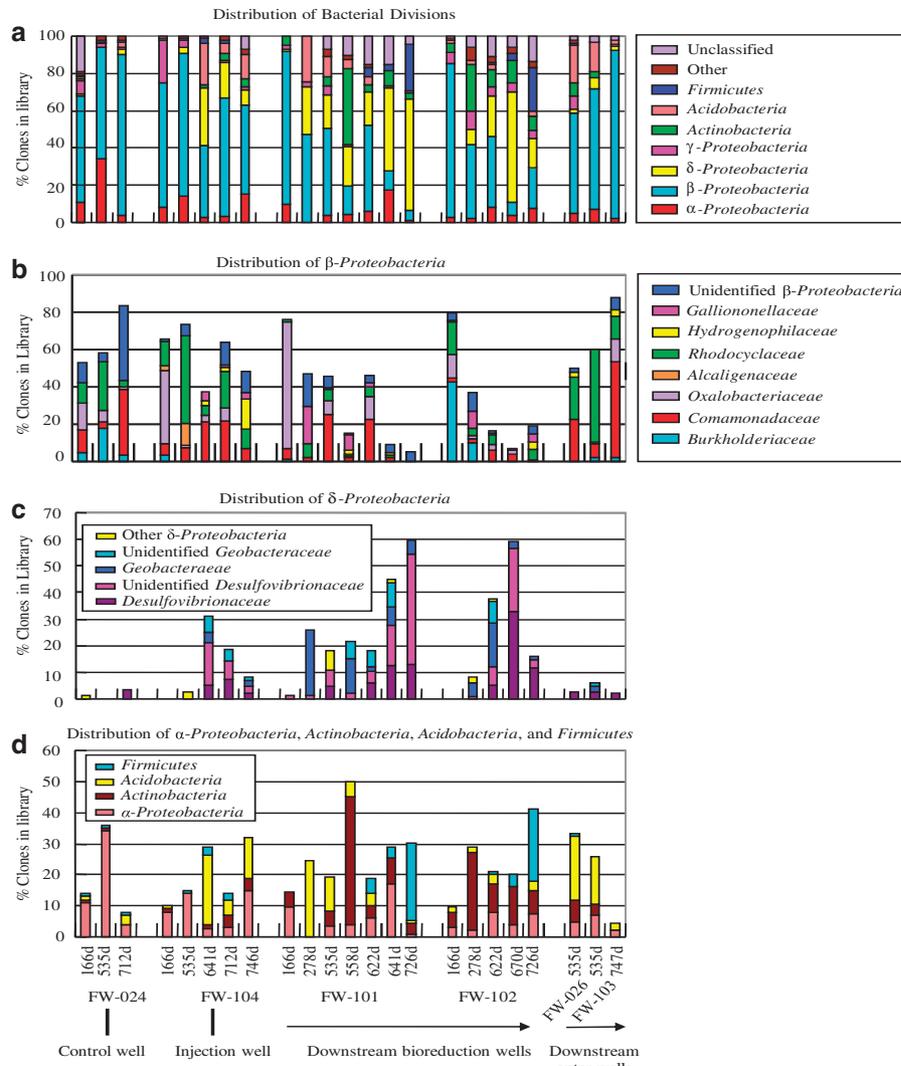

Figure 3 Distribution (% of the total) of clones from groundwater in each well over time according to all observed (**a**) bacterial divisions, (**b**) β-*Proteobacteria*, (**c**) δ-*Proteobacteria* and (**d**) α-*Proteobacteria*, *Actinobacteria*, *Acidobacteria* and *Firmicutes*.

closely related to an uncultivated *Thauera* spp. clone, DQ426920) in the uranium reduction phase before DO control (Figure 3b). The *Rhodocyclaceae* populations declined whereas *Comamonadaceae* populations increased (22%) during the initial phase of uranium reduction with DO control, and the majority of the clone library had 98% sequence identity to *R. ferrireducens* (Figure 3b and Supplementary Figure S2B). *Hydrogenophilaceae* populations increased (16%) by day 747 (Figure 3b) and the clone populations had ≥97% sequence identity to an uncultivated *Thiobacillus* sp. clone (EF413894) (Supplementary Figure S2A), and these results might coincide with the infiltration of oxygen.

FW101 shifted from predominance by *Oxalobacteraceae* (68%) to *Comamonadaceae* populations (25%) (Figure 3b), and the majority of the clone population had >97% sequence identity to *A. delafieldii* (Supplementary Figure S2C). *Gallionellaceae* populations were detected at 20% and 8% of the population at days 278 and 558, respectively, but were not detected once the well entered the phases of uranium reduction with DO control and ethanol limitation. Community dynamics in well FW102 was unlike that in FW101, with predominance by *Burkholderiaceae* (43%) and *Rhodocyclaceae* populations (17%) at day 166. Despite the differences in the bacterial community at day 166, the two wells had a similar overall community structure by days 641 and 622 for FW101 and FW102, respectively (Figure 3b).

After initiation of weekly ethanol additions, δ-*Proteobacteria* populations were detected by day 278 in wells FW101 and FW102 and by day 535 in FW104 (Figure 3c). Phylogenetic analysis of δ-*Proteobacteria* at the family level revealed differences in each well at the respective sampling days. However, *Desulfovibrionaceae* and uncultivated *Desulfovibrionaceae* populations predominated in both wells at the end of the experiment (Figures 3c and 4). For comparison, δ-*Proteobacteria* sequences were detected at <3% in FW024 (outer-loop injection well), FW103 and FW026 (down-gradient wells).

Although δ-*Proteobacteria* populations were predominant in both FW101 and FW102, the sampled communities within these wells changed over time (Figure 3a). The δ-*Proteobacteria* populations in FW101 shifted from predominance by *Geobacteraceae* populations (25%) during earlier stages of uranium reduction (day 278) to predominance by *Desulfovibrionaceae* during DO control (54%). *Geobacteraceae* were also detected in FW102 on day 278 (5%) and at a higher percentage on day 622 (25%) during the uranium reduction phase (no DO control), but declined at the later time points (<3%). *Desulfovibrionaceae* populations increased (to 56%) during the uranium reduction phase with DO control, but declined during the ethanol limitation phase (Figure 3c). The majority of *Desulfovibrionaceae* clones had 98% sequence identity to *Desulfo-*
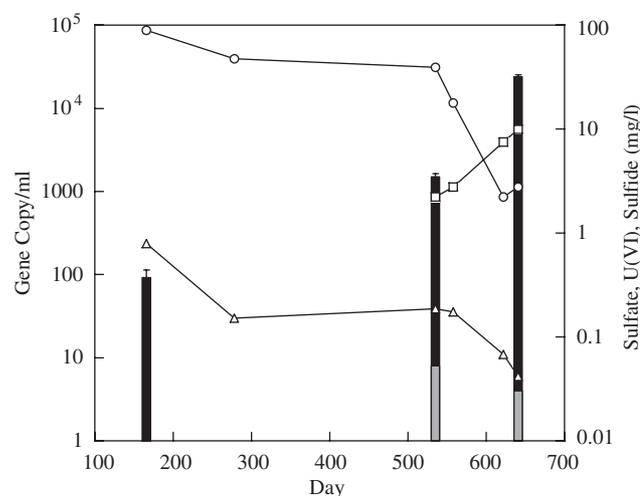

Figure 4 Gene copy number for *Desulfovibrio* (black bars) and *Geobacter* (gray bars) species based upon qPCR with conserved primers. The uranium (△), sulfate (○) and sulfide (□) levels over time in FW101. qPCR, quantitative PCR.

vibrio magneticus (D43994) (Figure 5). The majority of the *Geobacteraceae* clones had 100% sequence identity to an uncultivated *Geobacter* clone (AY780563) (Figure 5). To validate these results, qPCR was used to quantify *Desulfovibrio* spp. and *Geobacter* spp. from the same samples. The qPCR results demonstrated an increase in gene copy number for *Desulfovibrionaceae* (Figure 4), and a similar trend for these populations correlated to the clone library analyses ($r^2 = 0.9$).

Acidobacteria, Actinobacteria and α-*Proteobacteria* populations were more consistently observed for the tested wells during the time course, whereas *Firmicutes* populations were detected in certain wells at certain days (Figure 3d). The highest level of α-*Proteobacteria* (33% of clones) occurred in FW024 on day 535. The majority of α-*Proteobacteria* clones had 97% sequence identity to *Sphingobium herbacidovorans* (EF065102) (Supplementary Figure S3). *Sphingobium* sequences were detected earlier at an uncontaminated background site (Fields *et al.*, 2005).

Acidobacteria populations were detected at low levels in FW024 and FW102 at all time points. In FW101, *Acidobacteria* peaked on day 278 (25% of clones) and declined thereafter (Figure 3d). The majority of the clone populations had 97% sequence identity to *Geothrix fermentans* (U41563) (Supplementary Figure S4). *Actinobacteria* populations were consistently detected and represented 7% of the clones for FW026 on day 535 and lower levels at FW024, FW104 and FW103 throughout the experiment. Changes in the *Actinobacteria* population were especially pronounced in FW101 and FW102 (Figure 3d). The majority of these clones had 98% sequence identity to an uncultivated *Actinobacterium* clone (AJ888538) (Supplementary Figure S5). A study by Gremion *et al.* (2003) suggested that *Actinobacteria* populations were a major
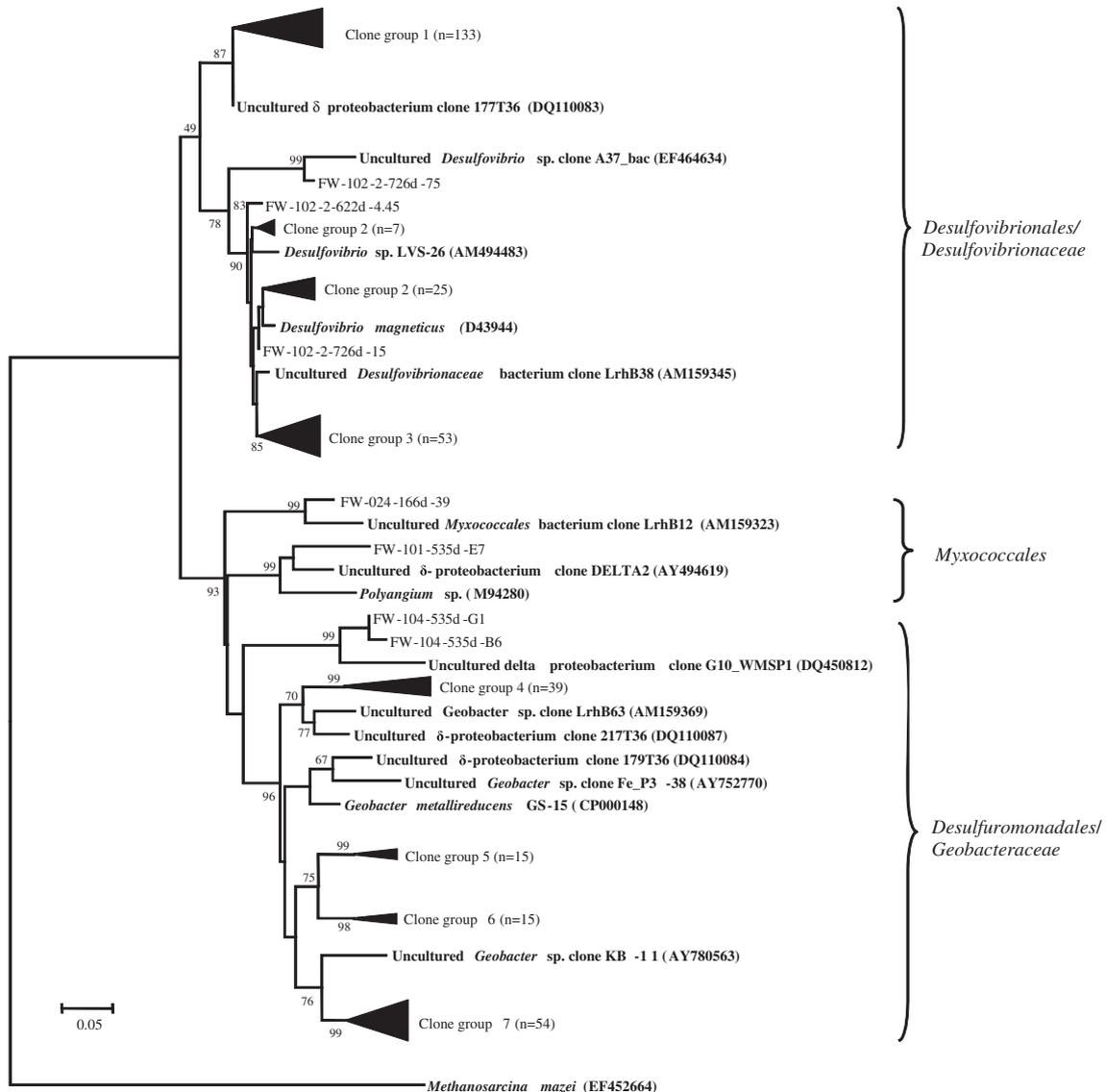
Figure 5 Phylogenetic relationship of cloned SSU rRNA genes classified in the δ-*Proteobacteria* and reference sequences (in bold) from GenBank (accession numbers in parentheses).

metabolically active group in a heavy –metal-contaminated soil.

Firmicutes were detected at <3% of clones between days 535 and 712 in FW104 and did not change significantly (<5% of clones). In both FW101 and FW102, however, *Firmicutes* populations increased to 25% during the ethanol limitation phase (days 713–754). The clone populations of the *Firmicutes* family in these two wells during this phase had 96–98% sequence identity to an uncultivated *Desulfosporosinus* spp. clone (AY607216) (Supplementary Figure S6).

Spatial and temporal analysis of the bacterial community
The temporal and spatial differences in the wells were further analyzed using hierarchical clustering with an UPGMA algorithm and principal coordi-

nates analysis (Figures 6a and b). The three wells most affected by groundwater recirculation and ethanol addition, FW104, FW101 and FW102, clustered together on day 166, which indicated that these wells were initially similar in community composition. The up-gradient well, FW024, did not have a major shift in bacterial community structure or composition over the tested time period. Moreover, the bacterial communities in the three wells FW104, FW101 and FW102 were similar to those of FW024 on day 166 (that is, before ethanol addition), which suggested that the initial communities were similar before the treatment strategy was implemented.

FW024 on day 712 clustered with FW103 on day 747 (>99.9% confidence) (Figure 6a). These two wells were the outermost up- and down-gradient wells, respectively. Similarly, the two down-gradient wells, FW026 and FW103, clustered together at
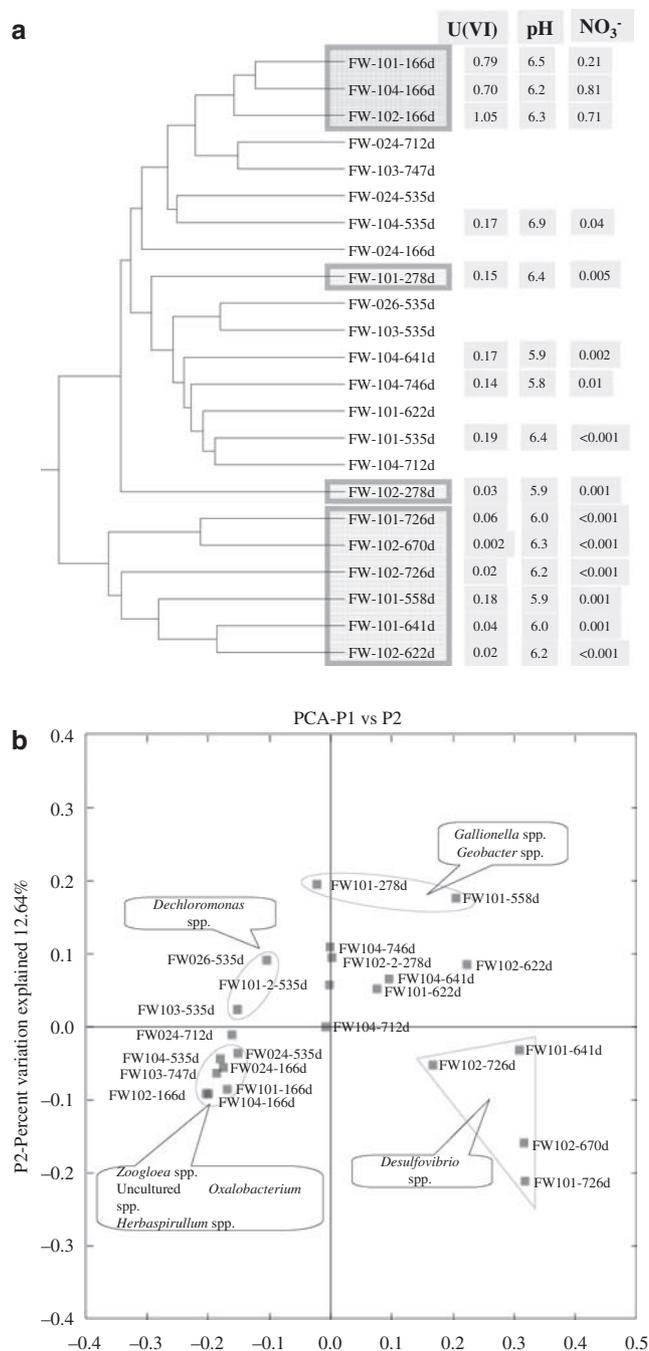
a

	U(VI)	pH	NO$_3^-$
FW-101-166d	0.79	6.5	0.21
FW-104-166d	0.70	6.2	0.81
FW-102-166d	1.05	6.3	0.71
FW-024-712d			
FW-103-747d			
FW-024-535d			
FW-104-535d	0.17	6.9	0.04
FW-024-166d			
FW-101-278d	0.15	6.4	0.005
FW-026-535d			
FW-103-535d			
FW-104-641d	0.17	5.9	0.002
FW-104-746d	0.14	5.8	0.01
FW-101-622d			
FW-101-535d	0.19	6.4	<0.001
FW-104-712d			
FW-102-278d	0.03	5.9	0.001
FW-101-726d	0.06	6.0	<0.001
FW-102-670d	0.002	6.3	<0.001
FW-102-726d	0.02	6.2	<0.001
FW-101-558d	0.18	5.9	0.001
FW-101-641d	0.04	6.0	0.001
FW-102-622d	0.02	6.2	<0.001

b

Figure 6 UniFrac statistical comparison of groundwater bacterial communities based upon phylogenetic comparisons. (**a**) UPGMA tree based upon UniFrac distances showing the overall phylogenetic relationships of each sampled community at a given sampling time. Nitrate, mM; U, mg l^{-1}. (**b**) Principal coordinate analysis (PCoA) of UniFrac distances resulted in a clustering pattern similar to that observed by UPGMA. The circled groupings indicate the bacterial communities that were grouped. Genus designations are based upon the population(s) in that community based upon lineage-specific analysis in UniFrac.

day 535 with >99.9% support. FW101 at days 278, 535 and 622 clustered with FW104 on days 641, 712 and 746. These data suggested that the injection

well lagged in bacterial community development compared with the communities at the downgradient monitoring wells that were most directly affected by ethanol addition. Community structure for monitoring wells FW101 and FW102 clustered at days 641 and 622 (90–99% confidence) and days 726 and 670 (99% confidence). These results indicated that the bacterial communities from the wells most directly impacted by ethanol addition were altered in different ways and ultimately became more similar to one another than the other wells.

Correspondence analysis resulted in similar results to the principal coordinates analysis that clustered the inner wells (Supplementary Figure S7). In addition, lineage-specific analyses revealed that clustering at earlier time points was because of the shared presence of several denitrifying bacterial populations. Samples from FW026 and FW103 at day 535 clustered because of the shared presence of both iron-oxidizing and iron-reducing populations, and samples from the two inner bioreduction wells clustered because of the shared presence of sulfate-reducing populations (Figure 6b). LIBSHUFF analyses of the injection and two biostimulation wells revealed similar results in which the bacterial communities developed changes in parallel while the injection well lagged ($P<0.001$) (data not shown). These results indicated that the bacterial communities within treated wells diverged sharply from those in up- and down-gradient wells, and that the community changes could be correlated to changes in geochemical conditions (for example, electron acceptors) during stimulation for bioremediation.

Bacterial community structure and groundwater variable relationships
When CCA was performed based upon population distributions with time and space as the variables, treatment wells (ethanol delivery) could be easily distinguished from outer- and inner-loop wells, and injection wells and treatment wells displayed a trajectory that followed a temporal vector (data not shown). CCA identified major species–environment correlations, and F-ratios were used to determine the variance for the independent variables as described earlier (ter Braak and Smilauer, 2002). The test of significance (global permutation tests) based upon all canonical eigenvalues ranged from 0.002 to 0.04 and indicated that the relations between the species and the measured environmental variables were highly significant.

When both the bacterial community structure and physicochemical variables were compared by pCCA, with engineering controls and geochemistry as variables and spatial location as covariables, the association of FW101 and FW102 at later time points was observed (Figures 7 and 8). There was a shift in community structure between times with
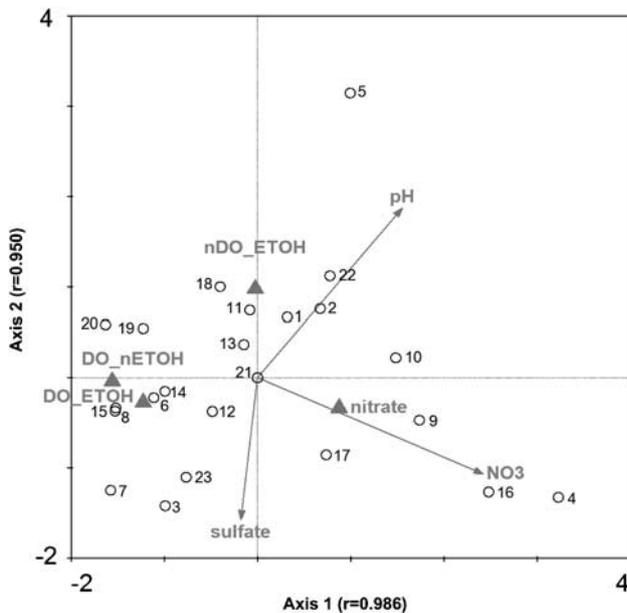
Figure 7 Ordination diagram from pCCA of % OTUs where engineering controls (nominal variables illustrated by triangles; nDO, no DO control; ETOH, ethanol was added; DO, DO was controlled; nETOH, no ethanol. DO, dissolved oxygen.) and subsurface geochemistry (quantitative variables illustrated by arrows) were environmental variables and spatial locations were covariables. Samples are represented as open circles and the numbers correspond to the sample number ID in Tables 1 and 2. The diagram accounts for approximately 33.5% of the variance in the species data ($P = 0.01$).

Figure 8 Ordination diagram of axes 1 and 2 of the bacterial families from the wells of the bioreduction zone where engineering controls (nominal variables illustrated by triangles) and subsurface geochemistry (quantitative variables illustrated by arrows) were environmental variables and spatial locations were covariables. 'Species' are illustrated by open triangles and the abbreviations are as follows: Oxal, *Oxalobacteraceae*; Brad, Bradyrhizobiaceae; Xant, *Xanthomonas*; Rhodos, *Rhodospirilla-ceae*; Burk, *Burkholderiaceae*; Rhiz, *Rhizobiaceae*; Alca, *Alcaligen-aceae*; Rhodoc, *Rhodocyclaceae*; Oth D, Other δ-*Proteobacteria*; Geob, *Geobacteraceae*; Bact, *Bacteroidetes*; Microb, *Microbacter-aceae*; Hydr, *Hydrogenophilaceae*; Clos, *Clostridiaceae*; Pept, *Peptococcaceae*; Caul, *Caulobacteraceae*; Myco, *Mycobacteraceae*; Desu, *Desulfovibrionaceae*; Alic, *Alicyclobacillaceae*; Phyl, *Phyllo-bacteraceae*; Microc, *Micrococcaceae*; Legi, *Legionellaceae*; Acti, *Actinobacteria*; Acid, *Acidobacteria*; Gall, *Gallionellaceae*; Ente, *Enterobacteraceae*; Pseu, *Pseudomonadaceae*. Only bacterial families with a cumulative fit ⩾5% on the first two axes are shown. Samples are illustrated by open circles and the numbers correspond to the sample ID number in Table 2. The diagram accounts for approximately 63% of the variance in the species data ($P = 0.018$).

and without DO control, irrespective of ethanol addition. Upon examination of the inner bioreduction wells only, the pCCA suggested that environmental variables (with space as a covariable) could explain approximately 63% of the species data ($P = 0.018$) (Table 3). U(VI), nitrate, COD, sulfide and sulfate strongly associated with the shifts in bacterial community patterns, and the correlations were meaningful in a biogeochemical context (for example, nitrate decline corresponded to decline in denitrifiers; sulfide correlated to the predominance of sulfate-reducing bacteria).

At day 166, FW104, 101 and 102 had high concentrations of nitrate and uranium and the associated bacterial communities were predominated by phyla common for nitrate-reducing bacteria (for example, β-*Proteobacteria* and γ-*Proteobacteria*) (Figure 8). Proceeding in a direction opposite to the gradient for nitrate and uranium indicates a negative correlation between nitrate and uranium and other bacterial communities. By contrast, nitrate-reducing species correlated positively to increasing COD, a measure of the available ethanol. The ordination diagram indicated that patterns of bacterial community were strongly associated with the spatial and temporal differences in the geochemistry of groundwater and that these markers could represent good indicators for radionuclide reduction potential and monitoring. For clarity, higher divisions of bacterial
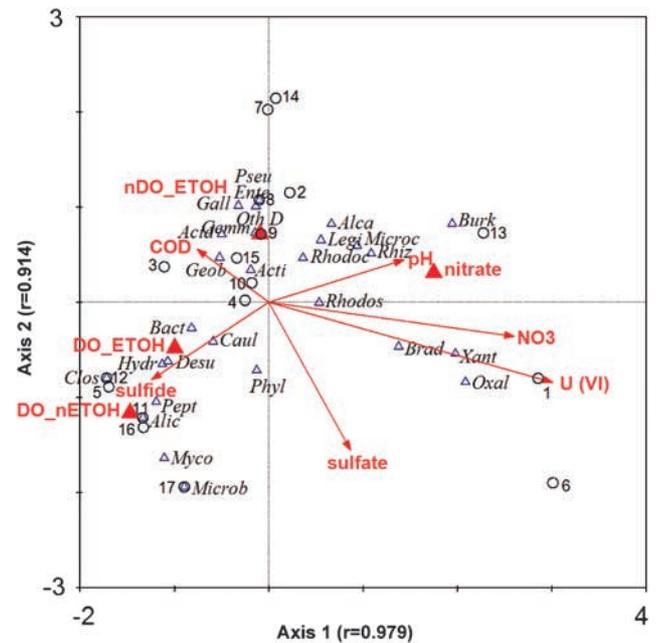
classification were used in the comparisons, and associations of particular populations could be discerned for the biostimulated wells. Thus, as the *Acidobacteria*, *Geobacteraceae*, *Actinobacteria*, *Desulfovibrionaceae* and *Firmicutes* populations increased, the levels of nitrate had declined and uranium declined. *Desulfovibrionaceae* and *Firmicutes* populations were also strongly associated with the sulfate (decline) and sulfide (increase) levels (Figure 8).

It is interesting to note that *Acidobacteria* and *Geobacteraceae* populations were positively correlated with higher COD levels (Figure 8). The results suggested that these populations were more prevalent when ethanol levels were higher during earlier and intermediate time points. In contrast, the sulfate reducing bacteria (SRBs) sequences (namely, *Desulfovibrionaceae*) predominated at later time points along with sequences for *Firmicutes*,

namely *Desulfosporosinus* spp. and uncultivated *Clostridium* species (Figure 8).

Samples that had a decrease in nitrate and U(VI) levels were clustered, and the phases of DO control with and without ethanol had similar community structure and composition (Figures 7 and 8). The relationships between the samples based upon community structure and the engineering controls were also highly significant ($P < 0.01$). However, the samples that represented time points when DO control was implemented compared with no DO control (with ethanol delivery) had a shift in community structure and the associated populations were *Desulfovibrio*, *Desulfosporosinus* and *Hydrogenophilaceae* species as well as *Bacteroidetes* and *Caulobacteraceae*. The pCCA data also suggested that the DO control phases had similar community structure and composition independent of ethanol addition. These results indicated that stimulant levels could be decreased or stopped at least for the tested time period without major shifts in population composition or distribution.

Total variation of the species data was partitioned into six categories (Table 3). When all wells were considered, the environmental (that is, engineering controls and subsurface geochemistry) and space variables accounted for the majority of variation (32.0% and 24.6%, respectively; Figure 9). However, 36% of the biological variation was unexplained (Figure 9). When only the biostimulated wells were considered, the environmental variable could account for approximately 50.1% of the variability, and the unexplained portion of the variability was 17.3% (Figure 9).

Discussion

Diversity and community function

Microbial communities have been extensively characterized at uranium-contaminated sites (Abdelouas *et al.*, 2000; Chang *et al.*, 2001, 2005; Anderson *et al.*, 2003; Elias *et al.*, 2003; Nevin *et al.*, 2003; Vrionis *et al.*, 2005) including the OR-FRC (Yan *et al.*, 2003; North *et al.*, 2004; Fields *et al.*, 2005, 2006; Akob *et al.*, 2007). In general, it is assumed that bacterial diversity will depend upon the degree of contamination and will change in structure and
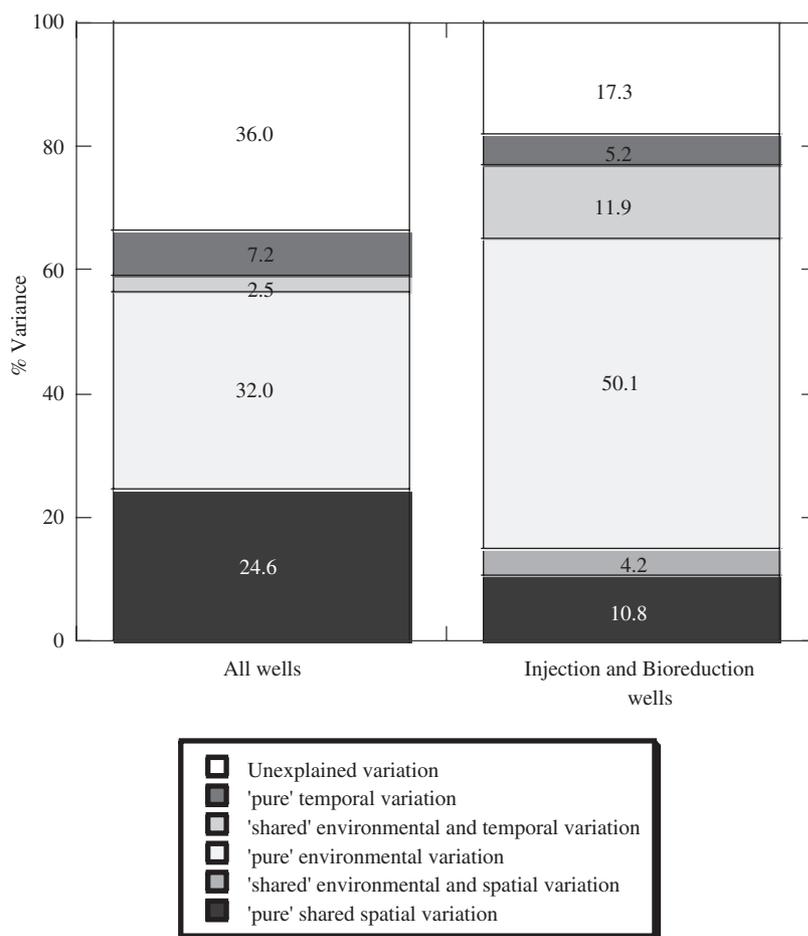

Figure 9 Partition of the total variation into six components: unexplained variation; 'pure' temporal variation; 'shared' variation between environment and time; 'pure' environmental variation; 'shared' variation between environment and space; and 'pure' spatial variation.

composition in response to geochemical changes (for example, nutrients, pH, E_h and so on). In our study, transitory trends were suggested from nitrate-reducing to iron-related redox metabolism to sulfate-reducing populations, and these results indicated that bacteria exhibited distributions at the landscape scale in agreement with predictable geochemical factors. During biostimulation, population distributions followed geochemical parameters (that is, nitrate to iron to sulfate reduction) with spatial differences that matched the expected geochemical changes; however, the biostimulated communities displayed idiosyncratic responses during the transitions (communities were dissimilar at intermediate time points). As described by Lawton et al. (1998), the idiosyncratic theory assumes that the relationship between species richness and ecosystem function does not follow a consistent pattern. In addition, the measured variables could only account for 50% of the biological variability in the stimulated wells.

The bioreduction wells (FW101 and FW102) had different streamlines passing from the injection well and progressed differently to a common community structure dominated by sulfate-reducing bacteria. The bioreduction wells displayed a variety of responses when diversity and uranium levels were compared as a measure of ecosystem process (that is, uranium reduction), and the comparison of nitrate levels to diversity displayed a similar relationship (Table 2; data not shown). The results suggested that the population distributions depended on the particular conditions of the local environment. Further work is needed to discern the relationships between specific ecosystem processes and bacterial diversity, but this study implied that population distributions depended on the particular conditions experienced as opposed to a system that is insensitive to diversity or one in which diversity contributes to ecosystem processes in a unique but predictable fashion.

The observed increase in bacterial diversity could be attributed to the input of energy and carbon source, which allowed increased colonization. The diversity of the treatment wells increased during the greatest decline in soluble U(VI) levels but fluctuated when U(VI) levels further decreased below $0.03\,mg\,l^{-1}$. The diversity increase was more likely a consequence of nutrient addition rather than the decline in U(VI) levels as uranium is not the only toxic contaminant present in the OR-FRC groundwater (Fields et al., 2006). The increase in diversity most likely altered population distributions, thereby changing the functionality of the community. It would appear that carbon/energy levels increased above a minimum level required for survival (or at least to the limit of detection) of various species. This type of response has been observed for plant diversity as productivity increases from very low levels (Huston and DeAngelis, 1994).

Engineering controls

Multivariate analytic tools are commonly used in macroecology studies to identify diversity and distribution patterns of plants and animals in relation to environmental parameters. Ramette (2007) suggested that these tools can also be readily applied in microbial ecology to help reduce data set complexity as DNA sequence data are increasingly available because of high-throughput sequencing technologies. Indeed, microbial ecology studies have increasingly used multivariate analyses to relate environmental variation with bacterial composition in aquatic systems (Yannarell and Triplett, 2005; Kent et al., 2007; Rubin and Leff, 2007), to determine bacterial community dynamics in relation to temporal variation in marine ecosystems (Pringault et al., 2007; Sapp et al., 2007), to develop microbial communities as indicators of ecosystem stress (Cao et al., 2006; Córdova-Kreylos et al., 2006; Fields et al., 2006), as well as to link geophysical signatures to microbial communities (Allen et al., 2007).

In our study, the use of pCCA enabled the identification of the crucial engineering controls with which bacterial community composition shifts appeared significantly related. We first compared bacterial community composition shifts between all wells and found that although the majority of the variation could be explained by spatial and environmental variables, a portion of the variance remained unexplained. Other ecological studies that used variance partitioning have also observed high levels of unexplained variation (Titeux et al., 2004). When we examined the bacterial community composition between the bioreduction wells only, the unexplained variation decreased. Titeux et al. (2004) suggested that the high unexplained species assemblages could be because of unaccounted factors such as the fluctuations of communities along temporal and spatial scales, unmeasured environmental variables and limitations in separating geographical factors that could be because of spatially structured processes. Our results suggested that other geochemical variables could be important indicators, and while it is difficult to measure everything, future efforts should attempt to include a range of variables to represent an even more holistic approach.

Changes in bacterial diversity were expected to result from engineering controls, and pCCA indicated that bacterial populations shifted with DO control measures and carbon/energy source additions. When ethanol was added, major shifts in sampled diversity and structure occurred, but when ethanol was withheld, DO control was needed to prevent further changes in the bacterial community. These results suggested that engineering controls impacted the development of bacterial communities with the desired metabolic activity. As pointed out by Kassen and Rainey (2004), microbiologists typically define function through mechanisms that involve genes and gene products, but a broadened perspective should include fitness of individual

populations. Fitness could be a measure of population survival and reproduction and is an ecological measure that relates genotype interactions with biotic and abiotic variables.

Other field studies observed bacterial population shifts during short-term push–pull stimulations, which were predominated by iron-reducing populations (North et al., 2004) or a mix of sulfate-reducing and/or iron-reducing populations (Abdelouas et al., 2000; Vrionis et al., 2005), whereas others observed predominance by sulfate reducing bacteria (SRBs), namely Desulfosporosinus (Nevin et al., 2003), Desulfosporosinus and Clostridium (Suzuki et al., 2003), Desulfobacter species (Vrionis et al., 2005). Over the tested period in our study, the communities became dominated by sulfate-reducing populations closely related to Desulfovibrio species. It is likely that additional populations are directly and/or indirectly involved in U(VI) bioreduction at this site, and it will become increasingly important to develop more sensitive molecular techniques as well as more robust cultivation methods to better understand remediation strategies.

Biotic associations
Similar to previous studies, we observed nitrate to be a major factor that influenced U(VI) reduction. After nitrate removal, a major shift in bacterial communities was observed with continued ethanol additions. Community structure first transitioned to nitrate reducing and then to populations of iron oxidizers (Gallionella ferruginea) and iron reducers (Geothrix spp., Geobacter spp., Holophaga spp.). G. ferruginea is able to live in low-oxygen conditions (Anderson and Pedersen, 2003) and has been found in many acidic metal-rich waters (Kim et al., 2002; Bruneel et al., 2006; Hallberg et al., 2006). Geothrix spp. have been isolated from a hydrocarbon-contaminated aquifer (Coates et al., 1999) and were also enriched when exposed to petroleum compounds in laboratory experiments (Abed et al., 2002). Geothrix-like sequences were also detected in the stimulated treatment zones of FRC sediments (Cardenas et al., 2008). Geobacter spp. have been found in metal-polluted freshwaters (Cummings et al., 2003) and detected in high numbers at sites treated for U(VI) remediation (Holmes et al., 2002; Anderson et al., 2003). The metabolic potential of iron-oxidizing and iron-reducing populations could have further implications on the stability of U(VI) immobilization as iron-oxidizing populations can potentially re-oxidize U(IV) and/or oxidize Fe(II) in a nitrate-dependent manner. The dynamics between iron oxidizers, iron reducers and sulfate reducers warrants further investigation at contaminated field sites.

Geobacter sequences were observed throughout the experiment but were highest when COD levels (that is, organic carbon) were elevated. The Geobacter populations appeared to associate with Acidobacteria populations (for example, Geotherix spp.,

and Holophaga spp.), but the nature of the possible interactions between these populations is unknown. Further work is needed to examine possible direct or indirect interactions. It is not known whether niche complementarity would lead to greater stability in bioremediation processes although studies have shown that it can explain positive relationships between diversity and function in prairie grasslands (Tilman et al., 1996).

Diverse Acidobacteria have been detected in different uranium-contaminated sites (Barns et al., 2007); however, little is known regarding physiology or ecology. The abundance of Acidobacteria in groundwater suggested an ability to tolerate high levels of metal and nitrate contamination, but the data also indicated that the Acidobacteria did not continue to compete with sulfate- and metal-reducing populations. Further analysis by pCCA revealed that the prevalence of Geobacter and Acidobacteria populations was more strongly correlated to the availability of ethanol rather than low U(VI) levels. These results emphasized the importance of a better understanding of population interactions leading to competition and/or mutually beneficial use of nutrient and energy sources.

In terms of the ecosystem process of interest in our study, multiple species could be classified into the same 'functional group' (that is, heavy metal reduction). For instance, both iron and sulfate reducers could be considered at least partially substitutable with respect to contribution to the ecosystem process of interest (that is, U(VI) reduction); however, iron and sulfate reducers can be very different organisms with respect to biogeochemistry and community interactions. Different studies have demonstrated the utility and predominance of iron and sulfate reducers at respective sites. Further work is still needed to determine how environmental conditions can be controlled at respective sites to optimize community structure, diversity and function within the context of desired ecosystem processes.

When DO control measures were compared with community composition, the results suggested that Geobacter spp. were more oxygen tolerant, as the populations were detected at higher levels when DO was not being controlled and that Desulfovibrio spp. were able to adapt to more anoxic conditions. An alternative explanation might be an interaction between Geobacter and an oxygen-tolerant microorganism. However, as mentioned earlier, the COD levels were also higher when DO was not specifically controlled. Separate studies at the OR-FRC showed that metal-reducing populations such as Geobacter spp. and Anaeromyxobacter spp. predominated in contaminated sediments following *in situ* biostimulation of microbial populations during push–pull tests (Petrie et al., 2003; North et al., 2004; Michalsen et al., 2007). However, different electron donors and engineering controls were used, and these comparisons also support the notion that

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there is an idiosyncratic relationship between population distribution and ecosystem process in terms of biostimulation for heavy metal reduction. As ethanol was being limited, *Firmicutes* were detected at increasing levels in groundwater and were especially pronounced in FW102. The *Firmicutes* sequences were closely related to the fermenting bacteria, *Clostridium* spp., the sulfate-reducing, *Desulfosporosinus* spp., and other sequences closely related to the *Peptococcaceae* family.

Research has shown that both *Clostridium* spp. and *Desulfosporosinus* spp. can be stimulated in uranium mine sediments (Suzuki *et al.*, 2003) and *Desulfosporosinus* spp. have also been enriched in high-salinity subsurface sediment stimulated for U(VI) reduction (Nevin *et al.*, 2003), as well as heavy metal column experiments (Geets *et al.*, 2006). *Clostridium* spp. were shown to be able to reduce U(VI) (Francis *et al.*, 1994; Madden *et al.*, 2007) and similar populations were also detected in other OR-FRC sediments using glycerol-amended enrichments (Petrie *et al.*, 2003). *Clostridium* populations were also suggested to be active members of the OR-FRC microbial community through a study of RNA-derived clone libraries (Akob *et al.*, 2007), and low-G + C bacteria have been isolated from OR-FRC groundwater (Fields *et al.*, 2005).

pCCA analysis indicated that bacterial communities were similar in the DO control phases regardless of electron donor availability. Indeed, *Desulfovibrio* populations were still predominant during this phase despite the fact that the subsurface condition may have started to favor fermentative metabolism by *Firmicutes*. The presence of populations that have both sulfate- and metal-reducing capabilities at the later phases of our study suggested a potential for continued uranium reduction during the periods when soluble electron donor is not provided. This was further observed by pCCA in which *Firmicutes* and *Desulfovibrio* populations were negatively correlated with U(VI) levels.

Although microbial communities adsorbed in the sediments can be significantly different from the planktonic populations in the aquifer, a recent study that characterized sediments at day 774 showed that *Desulfovibrio* spp. were the most abundant genus in the biostimulated zone (Cardenas *et al.*, 2008), and our results with groundwater also showed a predominance of *Desulfovibrio* sequences at this time period. The Cardenas *et al.* (2008) study also detected *Desulfosporosinus*, *Geobacter*, *Anaeromyxobacter*, *Geothrix* and *Acidovorax* related sequences associated with the sediment, and similar sequences were detected in our temporal study of the groundwater albeit at different proportions. These results suggested that some predominant populations might inhabit both the solid and aqueous phases (for example, *Desulfovibrio*), whereas others might be exclusive to solid phases (for example, *Acidobacteriaceae*). In the case of this test site, *Desulfovibrio* sequences predominated in both the sediments and

groundwater; however, it is not known whether the predominance of the same putative population(s) in the solid and aqueous phases would be unique to an engineered site and/or dependent upon the existing community composition. Although not the focus of this study, future work is needed to discern the importance and functionality of surface-adhered versus aqueous-phase communities.

Conclusion

The development of certain bacterial populations corresponded to the manipulated groundwater geochemical variables and showed that it is possible to stimulate the desired function in an engineered system. In terms of U(VI) immobilization, the wells responded differently, but prolonged engineering controls were able to stimulate growth of bacterial communities with the desired U(VI) reduction capacity. The notion of niche development through the application of selective pressures provides a possible framework for use of molecular ecology to improve process performance. Questions remain about how diversity and stability are related in terms of biochemical function, whether all *in situ* engineered systems would behave similarly, and whether the niche exclusion and selection concepts can be applied to improve models of active transport.

Acknowledgements

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